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Molecular typing of *Streptococcus agalactiae* isolates from fish

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Abstract

The genetic variability among *Streptococcus agalactiae* isolates recovered from fish was characterized using single-stranded conformation polymorphism (SSCP) analysis of the intergenic spacer region (ISR), and amplified fragment length polymorphism (AFLP) fingerprinting. A total of 46 *S. agalactiae* cultures isolated from different fish species and geographic origins as well as related reference strains were included in the study. ISR-SSCP divided the *S. agalactiae* isolates analysed into five distinct genotypes. Genotype 1 grouped all Kuwait isolates while genotype 4 clustered the majority of non-Kuwait isolates (USA, Brazil and Honduras). AFLP analysis offered a higher resolution level by dividing the isolates into 13 different genotypes. Two different AFLP profiles were identified within the Kuwait isolates. When data from both ISR-SSCP and AFLP were combined through a multidimensional analysis (MDS), a good correlation between geographical origin and genotypes was observed. Both AFLP and ISR-SSCP revealed genetic differences between *S. agalactiae* isolates from fish. While AFLP offered a higher resolution, ISR-SSCP also provided valid information being a simpler and faster method.

Keywords: amplified fragment length polymorphism, fish streptococcosis, genotyping, internal spacer region by single-strand conformation polymorphism, *Streptococcus agalactiae*.

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Introduction

Streptococcus agalactiae (group B streptococcus, GBS) is a major aetiological agent of septicaemia and meningoencephalitis in fish (Elliott, Facklam & Ritcher 1990; Eldar, Bejerano & Bercovier 1994; Eldar, Bejerano, Livoff, Horovitcz & Bercovier 1995; Evans, Klesius & Shoemaker 2006a). Among these fish species are Gulf killifish, *Fundulus grandis* Baird and Girard, hybrid striped bass, *Morone chrysops* × *Morone saxatilis*, *Morone chrysops* × *Morone saxatilis*, wild mullet, *Liza klunzinger* (Day), seabream, *Sparus aurata*, L., and Nile tilapia, *Oreochromis niloticus* (L.). Evans, Pasnik, Klesius & Al-Ablani (2006b) also reported the isolation of *S. agalactiae* from the muscle of a dead bottlenose dolphin, *Tursiops truncatus* Montague, from Kuwait. Furthermore, GBS is an important pathogen of terrestrial mammals including cows and humans (Dermer, Lee, Eggert & Few 2004). In cows, GBS causes bovine mastitis while in neonatal humans GBS causes sepsis, pneumonia, meningitis, osteomyelitis and soft tissue infections. A recent study supports the assertion that bovine and human GBS are largely unrelated (Bohnsack, Whiting, Bradford, Van Frank, Takahashi & Adderson 2004).

Diversity of *S. agalactiae* human and bovine isolates has been analysed using a broad range of genotyping methods including ribotyping, random amplified polymorphism, pulsed field gel electrophoresis (PFGE) and multilocus sequence typing (MLST). However, few or no DNA-based typing methods have been used to analyse *S. agalactiae* isolates from fish (Brochet, Couve, Zouine, Vallaeys, Rusniok, Lamy, Buschrieser, Trieu-Cuot, Kunst, Poyart & Glaser 2006). GBS-related morbidity and mortality in fish have occurred in the United

States (Louisiana and Mississippi), Honduras, Brazil and Ecuador (Evans *et al.* 2006a). The broad range of susceptible fish species and geographical distribution of *S. agalactiae* raise questions as to the role of GBS as an emergent pathogen in aquaculture. The objective of this study was to compare *S. agalactiae* isolates recovered from diseased fish to determine their genetic diversity in order to explore the epidemiology of GBS in aquatic environments. In this study, we genetically characterized a collection of *S. agalactiae* isolates from five fish species isolated from different geographical locations, as well as reference strains.

Materials and methods

Bacterial isolates

Forty-six *S. agalactiae* isolates from different sources, including eight reference strains from related species were used in this study (Table 1). All fish isolates have been identified as *S. agalactiae* based on standard biochemical methods (Evans, Klesius, Gilbert, Shoemaker, Al Sarawi, Landsberg, Duremdez, Al Marzouk & Al Zenki 2002). Stock suspensions of all isolates were stored in 10% glycerol at −70 °C. After thawing, the bacteria were grown in tryptic soy agar (TBS) for 24 h at 28 °C. Total DNA was extracted using the DNeasy Tissue Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions.

ISR-SSCP analysis

Streptococcus spp. specific primers corresponding to conserved regions in the 16S rRNA and 23S rRNA genes were used to amplify the internal spacer region (ISR) (McDonald, Fry & Deighton 2005). PCR conditions were as previously described (Arias, Welker, Shoemaker, Abernathy & Klesius 2004). Amplified ISR fragments were double digested using *Alu*I and *Hae*III (New England Biolabs, Ipswich, MA, USA) endonucleases following the manufacturer's instructions. Following digestion, restriction fragments were electrophoresed in a 3% agarose-1000 (Invitrogen, Carlsbad, CA, USA) gel for 90 min at 80 V, stained with ethidium bromide, and photographed under UV light. Single-strand conformation polymorphism (SSCP) analysis was carried out according to Olivares-Fuster, Shoemaker, Klesius & Arias (2006). One microlitre of restriction product was mixed with 5 μL of denaturing-loading solution (95% formamide, 0.025% bromophenol blue), heated for 5 min at 98 °C and immediately cooled on ice. All 6 μL of denatured products were separated by electrophoresis on GeneGel SSCP non-denaturing polyacrylamide gels (Amersham Biosciences, Piscataway, NJ, USA) hydrated with SSCP buffer A, pH 9.0 (Amersham Biosciences). A GenePhor electrophoresis unit (Amerham Biosciences) was used with the following running conditions: 5 °C constant temperature, 25 min at 90 V, 6 mA, 5 W plus 60 min at 500 V, 14 mA, 10 W. Gels were silver stained with the DNA Silver Staining Kit (Amersham Biosciences) and digitally photographed.

AFLP analysis

Amplified fragment length polymorphism fingerprints were determined as previously described by Arias, Verdonck, Swings, Garay & Aznar (1997). Briefly, 100 ng of total DNA was digested with 10 units of *Hin*dIII and *Taq*I (Promega, Madison, WI, USA) in a final volume of 30 μL. Following digestions, adapters were added to a final concentration of 0.04 and 0.4 μM for *Hin*dIII- and *Taq*I adapters, respectively, and ligated to the restriction fragments using T4 DNA ligase (Promega). AFLP® reactions employed two specific primers, oligonucleotide T000 (5′-CGATGAGTCCTGACCGAA-3′) corresponding to the *Taq*I ends, and H00A corresponding to the *Hin*dIII ends (5′-GACTGCGTACCAGCTTA-3′, selective base at the 3′-end is underlined). *Hin*dIII primer H00A was labelled with an IR700 fluorochrome from LI-COR (LI-COR, Lincoln, NE, USA). PCR conditions are described elsewhere (Arias *et al.* 1997). The PCR products were electrophoresed on a NEN Global Edition IR2 DNA Analyzer (LI-COR) following the manufacturer's instructions.

Data analysis

Calculation of similarity values and cluster analysis was done using BioNumerics software version 4.6 (Applied Maths, Sint-Martens-Latem, Belgium). Following conversion, normalization, and background subtraction with mathematical algorithms, levels of similarity between fingerprints were calculated with the Dice coefficient and the Pearson product-moment correlation coefficient (r) for SSCP-IRS and AFLP, respectively. Cluster analysis

Table 1 *Streptococcus agalactiae* fish isolates used in the study including American Type Culture Collection (ATCC) reference strains

Isolate	Host	Sample	Origin	Date	AFLP type	ISR-SSCP type
S. agalactiae ATCC 13813^T	Cattle	Milk	UK		3	2
S. agalactiae ATCC 12386	Cattle	Milk	USA		1	3
S. agalactiae ATCC 27956	Cattle	Milk	USA		1	2
S. agalactiae ATCC 31475	Human	CSF	USA		2	2
Streptococcus difficile ATCC 51487	Tilapia sp.	Brain	Israel		4	1
S. dysgalactiae ATCC 12388	Human	Throat	USA		12	6
S. dysgalactiae ATCC 12394	Human	Blood	Germany		13	6
Streptococcus iniae ARS-60	Hybrid striped bass	Brain	USA		11	7
S. agalactiae KU-MU-4-INT	Mullet	Intestine	Kuwait	2001	5	1
KU-MU-5BR	Mullet	Brain	Kuwait	2001	5	1
KU-MU-7-BR	Mullet	Brain	Kuwait	2001	5	1
KU-MU-12-BR	Mullet	Brain	Kuwait	2001	5	1
KU-MU-16-NARE	Mullet	Nare	Kuwait	2001	6	1
KU-MU-18-HK	Mullet	Head kidney	Kuwait	2001	5	1
KU-MU-20-HK	Mullet	Head kidney	Kuwait	2001	6	1
KU-MU-21-BR	Mullet	Brain	Kuwait	2001	5	1
KU-MU-22-EYE	Mullet	Eye	Kuwait	2001	6	1
KU-MU-25-HK	Mullet	Head kidney	Kuwait	2001	6	1
KU-MU-26-INT	Mullet	Intestine	Kuwait	2001	5	1
KU-MU-27-BR	Mullet	Brain	Kuwait	2001	5	1
KU-MU-28-HK	Mullet	Head kidney	Kuwait	2001	5	1
KU-MU-30-BL	Mullet	Blood	Kuwait	2001	5	1
KU-MU-35-EYE	Mullet	Eye	Kuwait	2001	5	1
KU-MU-36-BR	Mullet	Brain	Kuwait	2001	5	1
KU-MU-41-BL	Mullet	Blood	Kuwait	2001	5	1
KU-SB-1-BR	Seabream	Brain	Kuwait	2001	5	1
KU-SB-1-HK	Seabream	Head kidney	Kuwait	2001	5	1
KU-SB-1-EYE	Seabream	Eye	Kuwait	2001	5	1
KU-SB-37-BR	Seabream	Brain	Kuwait	2001	5	1
KU-SB-37-HK	Seabream	Head kidney	Kuwait	2001	5	1
KU-SB-38-BR	Seabream	Brain	Kuwait	2001	5	1
KU-DL-MUSCLE	Bottlenose dolphin	Muscle	Kuwait	2001	5	1
03ARS-BZ-TN-01	Nile tilapia	Brain	Lavras Parana, Brazil	2003	4	1
03ARS-BZ-TN-04	Nile tilapia	Brain	Lavras Parana, Brazil	2003	7	4
03ARS-BZ-TN-05	Nile tilapia	Brain	Lavras Parana, Brazil	2003	7	4
03ARS-BZ-TN-06	Nile tilapia	Brain	Lavras Parana, Brazil	2003	7	4
03ARS-BZ-TN-09	Nile tilapia	Brain	Lavras Parana, Brazil	2003	7	4
LADL-90-503	Hybrid striped bass	Brain	Louisiana, USA	1990	7	4
LADL-97-151	Gulf killifish	Brain	Louisiana, USA	1997	7	4
LADL-00-192	Gulf killifish	Brain	Louisiana, USA	2000	7	4
LADL-00-351a	Nile tilapia	Brain	Mississippi, USA	2000	7	4
LADL-02-097	Gulf killifish	Brain	Louisiana, USA	2002	7	4
LADL-05-108a	Nile tilapia	Brain	Honduras	2005	10	4
IS-ET-09-03	Hybrid striped bass	Brain	Israel	2003	9	5
04ARS-BZ-TN-002	Nile tilapia	Brain	Lavras, Brazil	2004	7	4
04ARS-BZ-TN-003	Nile tilapia	Brain	Lavras, Brazil	2004	8	4
04ARS-BZ-TN-004	Nile tilapia	Brain	Lavras, Brazil	2004	7	4
04ARS-BZ-TN-005	Nile tilapia	Brain	Lavras, Brazil	2004	9	4
04ARS-BZ-TN-006	Nile tilapia	Brain	Lavras, Brazil	2004	7	4

AFLP, amplified fragment length polymorphism; ISR-SSCP, intergenic spacer region by single-strand conformation polymorphism.

was performed with the unweighted pair-group method using average linkages (UPGMA).

The index of discriminatory power (D) for each typing method was calculated based on Simpson's diversity index (Hunter 1990; Kuhn, Austin, Austin, Blanch, Grimont, Jofre, Kovabli, Larsen, Mollby, Pedersen, Tiainent, Verdonck & Swings 1996).The concordance between AFLP and ISR-SSCP was determined using BioNumerics by comparing the values from the similarity matrices of the typing methods that were plotted on *x*- and *y*-axes. This graph provides an indication of the degree of concordance between the two techniques. The Kendall's τ correlation coefficient between both methods was also calculated.

Results

ISR-SSCP analysis

Amplification of the ISR sequence was successfully achieved from all fish isolates. Amplified products were approximately 1500 bp. After restriction and SSCP analysis, *S. agalactiae* isolates were divided into five distinct ISR-SSCP profiles (Table 1) at 80% similarity (dendrogram not shown). The *S. dysgalactiae* and *S. iniae* reference strains could be clearly separated from *S. agalactiae* by this method. ISR-SSCP profiles obtained in the study are shown in Fig. 1. The ISR-SSCP-1 group clustered all isolates from Kuwait Bay, regardless of fish species, and one isolate from Brazil (03ARS-BZ-TN-01). The remainder of isolates (including Brazil, Honduras and USA isolates) shared a highly similar profile and were grouped as ISR-SSCP-4. *Streptococcus agalactiae* reference strains clustered apart from fish isolates showing two different ISR-SSCP profiles (ISR-SSCP-2 and 3). Finally, Israeli isolate (IS-ET-03) presented the unique profile ISR-SSCP-5.

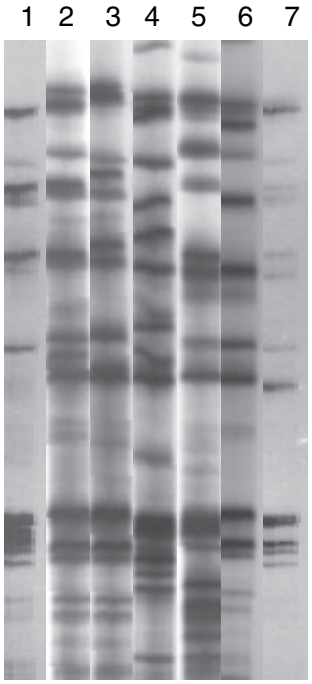

Figure 1 Representative genotypes of *Streptococcus agalactiae* based on intergenic spacer region by single-strand conformation polymorphism (ISR-SSCP) profiles. Lane 1, genotype ISR-SSCP-1, isolate KU-MU-27-BR; lane 2, genotype ISR-SSCP-2, isolate ATCC 13813; lane 3, genotype ISR-SSCP-3, isolate ATCC 12386; lane 4, genotype ISR-SSCP-4, isolate 04ARS-BZ-TN-006; lane 5, genotype ISR-SSCP-5, isolate IS-ET-03; lane 6, genotype ISR-SSCP-6, isolate ATCC 12388; lane 7, genotype ISR-SSCP-7, isolate ARS-60.

AFLP analysis

Figure 2 shows the results of the cluster analysis of the AFLP patterns generated in the study. It is noteworthy that this technique yielded isolate-specific patterns of 65–80 distinct bands ranging from 100 to 900 bp (because of the molecular marker used for normalization only bands below 700 bp were included in the analysis). As with ISR-SSCP analysis, *S. dysgalactiae* and *S. iniae* presented very different fingerprints, sharing less than 20% similarity with *S. agalactiae*. Up to 10 different AFLP types were defined within the *S. agalactiae* species at 85% similarity. Reference strains clustered apart from fish isolates in groups 1, 2, 3 and 4. The only exception was reference strain ATCC 51487 that shared a 90% similarity with environmental isolate 03ARS-BZ-TN-01. Kuwait isolates (24) were divided into two AFLP types (5 and 6). Group 5 contained isolates from mullet and sea bream while group 6 contained only mullet isolates.

Comparison of ISR-SSCP and AFLP

The discriminatory powers as expressed by Simpson's index of diversity were 0.582 for ISR-SSCP and 0.811 for AFLP. As all isolates could be typed, the typeability for both methods was 1. When plotting the pair-wise comparison of isolates typed with ISR-SSCP against the pair-wise comparison of isolates typed with AFLP in an *x*- and *y*-graph, a good correlation was observed. Kendall's τ correlation coefficient was 0.68. When data from both (ISR-SSCP and AFLP) methods were combined as a composite set thereby creating a new similarity matrix, this divided the *S. agalactiae* population into three groups. Figure 3 shows the spatial distribution of these groups in a multi-dimensional scaling plot (MDS) and their correspondence with geographical origin. All Kuwait isolates clustered together forming a tight group. A second group was formed by all *S. agalactiae* reference strains (including *S. difficile* ATCC 51487) and Brazilian isolate 03ARS-BZ-TN-01. All American isolates clustered apart and a third group could be inferred. The Israeli isolate IS-ET-09-03 could not be ascribed to any group based on MDS analysis.

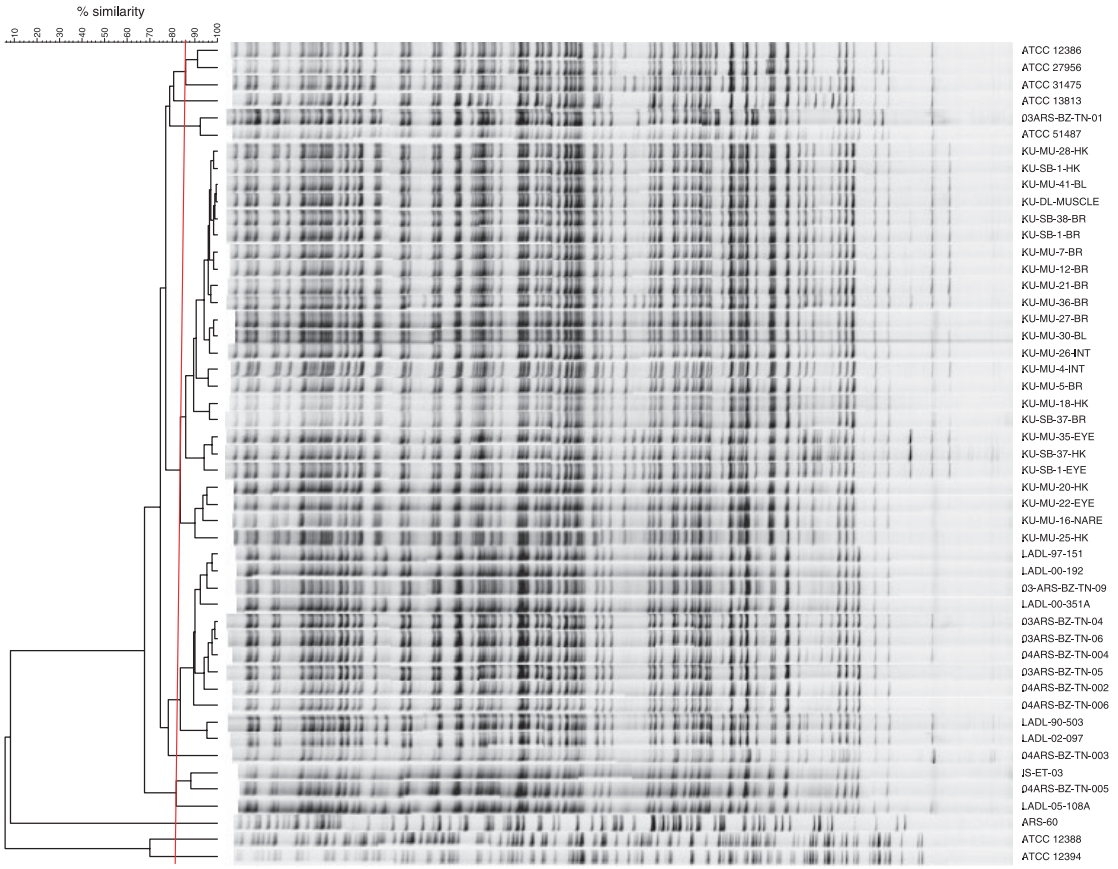

Figure 2 Amplified fragment length polymorphism (AFLP) patterns of *Streptococcus agalactiae*. The dendrogram was derived by unweighted pair-group method using average linkages cluster analysis of the AFLP profiles of 46 *S. agalactiae* strains and reference strains. The tracks show the processed band patterns after conversion, normalization and background subtraction. Levels of linkage are expressed as the Pearson product-moment similarity coefficient. Red line indicates the cut-off point for AFLP-type definition at 87%.

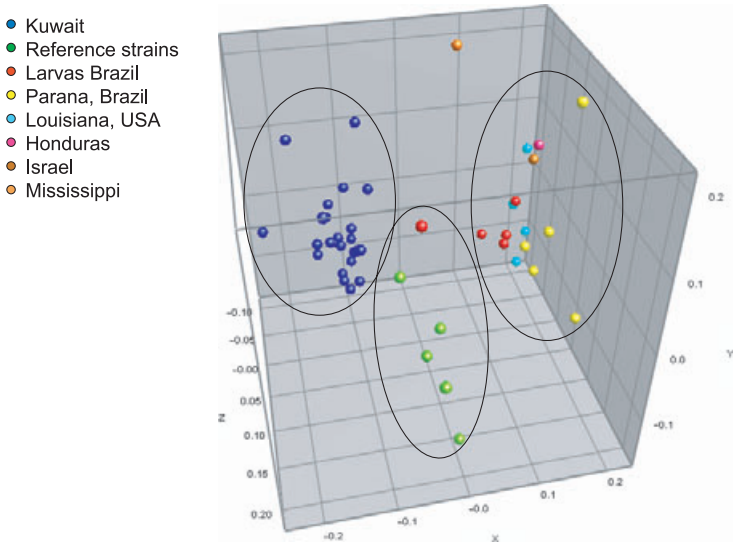

Figure 3 Multidimensional scaling plot using the similarity matrix obtained by combination of intergenic spacer region by single-strand conformation polymorphism (ISR-SSCP) and amplified fragment length polymorphism (AFLP) data. Only *Streptococcus agalactiae* (and *Streptococcus difficile* ATCC 51487) isolates are shown in this analysis. Geographic origin is represented by colour coding. Clusters defined by ISR-SSCP and AFLP are marked by circles.

Discussion

Intraspecies characterization of *S. agalactiae* has been focused on human and bovine isolates (Kong, Martin, James & Gilbert 2003; Kawamura, Itoh, Mishima, Ohkusu, Kasai & Ezaki 2005; Brochet *et al.* 2006). Several methods including serotyping, multilocus enzyme electrophoresis (MLEE), ribotyping, PFGE and MLST have been used for strain typing of *S. agalactiae* (Brochet *et al.* 2006). The majority of these methods separated human from bovine isolates although only MSLT was able to infer phylogenetic relationships between clones. Although *S. agalactiae* has been identified as the causal agent of meningoencephalitis in natural and farmed fish populations, little information is known about the genetic diversity of these fish isolates as well as their genetic relatedness to reference strains. Brochet *et al.* (2006) extensively characterized more than 70 strains of *S. agalactiae* using different genetic methods. Only one fish isolate was included in that study but it was the most dissimilar strain among all the isolates tested.

Genetic characterization of *S. agalactiae* isolates from fish separated them from human and bovine reference strains with only two exceptions. First, the reference strain ATCC 51487 [isolated from tilapia and originally classified as *S. difficile*, a later synonym of *S. agalactiae* (Kawamura *et al.* 2005)] was genetically similar to human and bovine isolates, including the type strain ATCC 13813. In fact, strain ATCC 51487 was closely related to another tilapia isolate (03ARS-BZ-TN-01) by both fingerprinting methods used and to Kuwait isolates by ISR-SSCP. The rest of the fish isolates were divided into two main genetic clusters corroborated by ISR-SSCP and AFLP. All Kuwait isolates belonged to the same group and were indistinguishable by ISR-SSCP. However, although forming a tight cluster by AFLP fingerprinting analysis, subgroups could be observed. Unfortunately, these subgroups could not be correlated to fish species, or tissue of isolation. Some mullet and seabream isolates showed an extremely high similarity by AFLP (≥97%) and under the conditions of our analysis (Arias *et al.* 2004) could not be distinguished (i.e. KU-MU-35-EYE and KU-SB-37-HK). Interestingly, some seabream isolates recovered from the same fish but from different organs (i.e. KU-SB-1-HK, KU-SB-1-EYE, KU-SB-37-HK and KU-SB-37-BR) were genetically distinct (at 87% similarity although slightly above the cut-off point chosen at 85%). Moreover, all AFLP types among Kuwait isolates shared similarities above 85% and appeared intertwined in the dendrogram analysis regardless of fish species. These findings suggest that the 2001 outbreak of *S. agalactiae* in Kuwait Bay (Evans *et al.* 2002) was caused by a concurrent polyclonal infection. With our current data, we cannot conclude whether wild mullet or cultured seabream was the original source for the pathogen.

American isolates, including representatives from North, Central and South America, formed a robust genomic cluster supported by both methods and could be clearly differentiated from the Kuwait group. Some subgroups could also be observed when using AFLP but no clear correlation between genotype and geographical origin or fish species could be inferred.

Both ISR-SSCP and AFLP allowed us to reveal the genetic diversity of *S. agalactiae* isolates recovered from fish. In addition, similar species within the genus were clearly separated from *S. agalactiae*. More importantly, both fingerprinting methods were in agreement, splitting the isolates into two main genetic clusters that could be correlated with geographical origin or outbreak (as all Kuwait isolates came from a single infectious episode correlation to outbreak and not geographical origin is intended). As was expected, AFLP offered a higher resolution providing a higher diversity index for the species. However, taking into account the resolution power, cost, labour and time, ISR-SSCP analysis could be a good tool for routine characterization of *S. agalactiae*.

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